Identification of Amino Acid Residues Involved in Substrate Recognition by the Catalytic Subunit of Bovine Cyclic AMP Dependent Protein Kinase: Peptide-Based Affinity Labels†

Shahriar Mobashery and E. T. Kaiser*

Laboratory of Bioorganic Chemistry and Biochemistry, The Rockefeller University, New York, New York 10021-6399

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ABSTRACT: Two peptide-based affinity inactivators Ac-Leu-(BrAc)Orn-Arg-Ala-Ser-Leu-Gly (**4**) and Ac-Leu-Arg-(BrAc)Orn-Ala-Ser-Leu-Gly (**5**) were prepared as probes for the study of the nature of the active-site residues in the catalytic subunit of cyclic AMP dependent protein kinase. Under conditions of inhibitor in excess, both peptides inactivated the catalytic subunit by an apparent biphasic process. A fast phase, which inactivated the protein by ~40%, was followed by a slow phase that accounted for the loss of the remaining enzyme activity. Protection experiments with the kinase substrates showed that the slow phase of inactivation was active site directed, while the fast phase was not. Studies with radioactively labeled peptides **4** and **5** indicated incorporation of two peptide residues per molecule of the catalytic subunit upon complete inactivation. This observation is consistent with the occurrence of one alkylation event in each phase of the inactivation. The protein was proteolyzed subsequent to its modification with radioactive peptides. High-performance liquid chromatography afforded two radioactive peptide fragments in each case, which were sequenced by Edman degradation. Peptide **4** alkylated Thr-197 and Glu-346, while peptide **5** modified Cys-199 and also Glu-346. Data are presented to support the conclusion that Thr-197 and Cys-199 are located at or near the active site.

Adenosine cyclic 3′,5′-phosphate dependent protein kinase (cAMP kinase) is an important eukaryotic regulatory enzyme. The holoenzyme, consisting of four subunits, dissociates in the presence of cAMP to give cAMP bound to the regulatory subunit dimer and two liberated catalytic subunits. Two classes of cAMP-dependent protein kinases (types I and II) have been identified that appear to differ only in their regulatory subunit (Hofmann et al., 1975). The catalytic subunit has been shown to be responsible for the phosphorylation of serine and threonine residues in specific protein substrates, the physiological functions of which are modulated by the kinase's phosphotransferase activity. The literature on cAMP-dependent protein kinases has been the subject of a recent review (Bramson et al., 1983).

Studies of peptide sequences in natural protein substrates that undergo phosphorylation, in conjunction with related studies on synthetic oligopeptides, have identified the preferred primary amino acid sequences for kinase substrates. A prototypic peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly (**1**), that corresponds closely to the phosphorylation site in porcine liver pyruvate kinase (Hjelmquist et al., 1974) is phosphorylated at the serine hydroxyl by the catalytic subunit. The kinetic parameters (V_{max} = 20 μmol min^{-1} mg^{-1} and $K_{m,peptide}$ = 16 μM) compare favorably to those for the phosphorylation of natural substrates by the same enzyme (Kemp et al., 1977). In collaboration with Mildvan and colleagues, we investigated the conformations of peptide **1** and $Mg^{II}ATP$ in the kinase active site by NMR experiments (Granot et al., 1979; Rosevear et al., 1983). Our data indicated an anti conformation for the metal–nucleotide complex when bound to the enzyme active site. Additionally, NMR studies on the active site bound peptide **1** suggested extended coil conformations for the peptide (Granot et al., 1981). The conformation of the reactive peptide in the active site of the catalytic subunit has been defined further by recent studies on the reactivity of depsipeptide analogues and N-methylated analogues of peptide **1** (Bramson et al., 1987).

While much has been learned concerning the conformational arrangements of peptide substrates and metal–nucleotide complexes at the active site, a paucity of information exists regarding the crucial active-site residues involved in substrate recognition and/or catalysis. This information is essential for the elucidation of the mechanism by which catalysis occurs.

Many chemical modifications of the catalytic subunit have been reported [for references, see Bramson et al. (1983)]. However, only two substrate-based affinity inactivators, one an ATP analogue and the other a peptide **1** analogue, have been studied thoroughly with this enzyme. By this approach lysine-72 (Zoller et al., 1981) and cysteine-199 (Bramson et al., 1982) have been shown to be at or near the active site. To probe further the nature of other active-site amino acid residues, we have substituted systematically amino acid residues in the primary sequence of peptide **1** by amino acid analogues that can act as electrophiles. Since peptide **1** has an extended coil conformation at the active site, it seemed likely that it could act as a sort of "molecular ruler". By moving the electrophilic amino acid analogues along the peptide chain, we could obtain a series of peptide-based affinity labels that would map different active-site recognition residues. Initially, we decided to make substitutions at the two arginine residues in compound **1**. The requirement for the two arginine residues for substrate recognition has been documented for a large number of synthetic and natural substrates (Kemp et al., 1975; Bramson et al., 1983). In addition, from circular dichroism experiments it has been suggested that the enzyme interacts initially with the substrate by recognition of the Arg–Arg sequence on the peptide—presumably by its N-terminal acidic domain (residues 328–350)—concomitant with a conforma-

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tional change (Reed et al., 1985). We report here our results on probing the recognition site of the catalytic subunit of cAMP-dependent protein kinase with two affinity analogues based on the primary sequence of peptide **1**, where each arginine residue has been replaced with N^{δ}-(bromoacetyl)-ornithine. These peptides modify the catalytic subunit in an active-site-directed process.

Materials and Methods

Visible spectrophotometric analyses were performed by using a Perkin-Elmer Lambda-5 spectrophotometer. Radioactive decay was measured on a Beckman LS 7000 scintillation counter. Mass spectra were obtained on a VG7070-EQ instrument at the Columbia University Mass Spectrometry Facility. Xenon was used as the primary beam of fast atoms at an accelerating voltage of 5 kV. For mass spectrometry, samples were dissolved in DMSO/H_2O/glycerol/thioglycerol matrix (5:5:1:1). Amino acid analyses were carried out by using a Beckman Model 121 amino acid analyzer according to the method of Moore and Stein (1963) after hydrolysis of the peptide in 6 N constant boiling HCl (Pierce) at 110 °C for 24 h. High-performance liquid chromatography was carried out on a Perkin-Elmer Series 410 system, and effluent was monitored by a Perkin-Elmer variable-wavelength LC 75 detector.

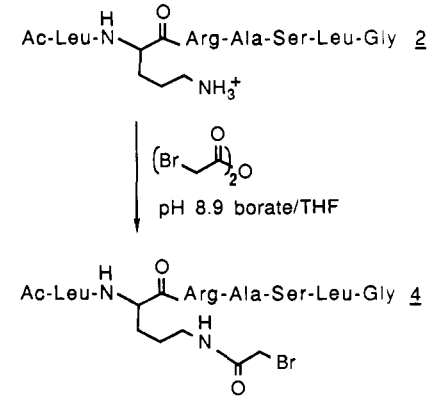
The catalytic subunit of the cAMP-dependent protein kinase was purified to homogeneity from bovine heart as previously described (Bramson et al., 1982). [1-^{14}C]Bromoacetic acid was purchased from Amersham. Bromoacetic acid was an Aldrich product. Bachem Inc. was the supplier of α-Boc-δ-Cbz-L-ornithine, while all the rest of the Boc-amino acids were obtained from Peninsula Laboratories Inc. Adenosine 5′-(β,γ-methylenetriphosphate) and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin were purchased from Sigma Chemical Co. *Staphylococcus aureus* V8 protease was obtained from Cooper Biomedical.

Preparation of Ac-Leu-Orn-Arg-Ala-Ser-Leu-Gly (**2**) *and Ac-Leu-Arg-Orn-Ala-Ser-Leu-Gly* (**3**). Stepwise syntheses were carried out by using the Merrifield resin (Merrifield, 1963). Boc-Gly was attached to the resin by the method of Horiki et al. (1978). N^{α}-Butoxycarbonyl groups were removed by a 20-min mixing in 50% trifluoroacetic acid (TFA) in CH_2Cl_2, followed by neutralization in 5% diisopropylethylamine (DIEA) in CH_2Cl_2 and coupling with symmetric N^{α}-Boc-amino acid anhydrides (3 equiv). The completion of each coupling step was monitored with ninhydrin (Kaiser et al., 1970). The terminal amino groups of the peptides were acetylated in CH_2Cl_2 by using a 10-fold excess of acetic anhydride. The peptides were deprotected and removed from the resin by treatment with HF in the presence of anisole (0 °C, 1 h) according to the method of Sakakibara et al. (1967). Preparative HPLC purification of the crude peptides followed by lyophilization afforded the desired heptapeptides as fluffy, white solids.

Ac-Leu-Orn-Arg-Ala-Ser-Leu-Gly (**2**): MS (FAB$^+$), m/z 772 (45%, M + H), 794 (30%, M + Na); amino acid analysis Ser (1.02), Gly (0.99), Ala (1.00), Leu (1.80), Orn (1.00), Arg (1.10); HPLC single peak t_R = 15.0 min (Altex, Ultrasphere ODS, 1.0 × 25 cm, 5–50% linear acetonitrile gradient in 150 mM $NaClO_4$, 50 mM NaP_i, pH 2.6, over 30 min, 3 mL/min, 225 nm).

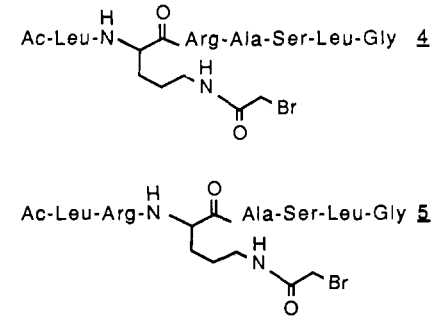
Ac-Leu-Arg-Orn-Ala-Ser-Leu-Gly (**3**): MS (FAB$^+$), m/z 772 (35%, M + H), 794 (20%, M + Na); amino acid analysis Ser (0.93), Gly (1.00), Ala (1.00), Leu (1.80), Orn (0.95), Arg (0.99); HPLC single peak t_R = 13.0 min (conditions as described for **2**).

Preparation of the Bromoacetyl Peptides **4** *and* **5**. A solution of bromoacetic acid (19 mg, 137 μmol) in 0.5 mL of tetrahydrofuran (THF) was allowed to react with a 1 M solution of dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 (65 μL) at room temperature in the dark. After 15 min, a solution of peptide **2** or **3** (5.0 mg, 6.5 μmol) in 1 mL of sodium borate



buffer (250 mM, pH 8.9) was added, and the resulting solution was stirred vigorously for 20 min. The completion of the reaction was verified by a negative ninhydrin test. The resulting suspension was passed through a glass-wool plug in a Pasteur pipet, followed by filtration through a 0.2-μm filter unit (Uniflo). The peptide was subsequently purified by HPLC (Altex, Ultrasphere ODS, 1.0 × 25 cm) by using a 5–80% linear acetonitrile gradient in 0.1% TFA. The bromoacetylated peptides eluded at $t_R \simeq 12.0$ min as monitored by absorbance at 230 nm. Acetonitrile was evaporated in vacuo at room temperature, and the aqueous solution was lyophilized to give the desired product as a white powder. Yields were typically 60–65%.

Radioactive peptides were prepared by the same procedure. Typically, [1-^{14}C]bromoacetic acid (250 μCi) was diluted with 18 mg of unlabeled bromoacetic acid prior to the DCC-mediated preparation of the symmetric anhydride. After purification, radioactive peptides with specific activities of ~2000 dpm/nmol were obtained.



Peptide **4**: MS (FAB$^+$), m/z 814 (30%, M − Br), 892, 894 (45%, M + H); HPLC single peak, t_R = 12.0 min (Altex, Ultrasphere ODS, 1.0 × 25 cm, 50–80% linear acetonitrile gradient in 0.1% TFA over 20 min, 3 mL/min, 225 nm); positive Sakaguchi test for the presence of unsubstituted guanidine (Sakaguchi, 1925); negative ninhydrin test for the absence of primary amine.

Peptide **5**: MS (FAB$^+$), m/z 814 (20%, M − Br), 892, 894 (30%, M + H); HPLC single peak, t_R = 12.0 min (conditions as described for peptide **4**); positive Sakaguchi test; negative ninhydrin test.

Assay of Enzymatic Activity. The spectrophotometric method developed by Bramson et al. (1980) was used to assay enzyme activity. This approach utilizes the chromogenic peptide Leu-Arg-Arg-(o-NO_2)Tyr-Ser-Leu-Gly, which un-

dergoes a spectral change upon phosphorylation at 430 nm, allowing for continuous monitoring of this reaction. The assay was carried out at 35 °C in 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer, pH 7.0, containing 10 mM $MgCl_2$, 150 mM KCl, 0.2 mM dithiothreitol (DTT), and 0.2 mg/mL bovine serum albumin. A typical 1.0-mL assay mixture contained 100 μM chromogenic peptide, 2.0 mM ATP, and 10–15 nM enzyme.

Inactivation assays were performed in the following manner. A solution of a given peptide inactivator in 50 mM MOPS containing 1 M KBr (pH 7.6) was incubated at 35 °C. Enzyme was added to give a final concentration of 10 μM. At various time intervals 5-μL aliquots of the inactivation mixture were removed and diluted into 495 μL of 0.2 mg/mL bovine serum albumin. A 100-μL portion of the resultant solution was mixed with 900 μL of assay mixture, and the activity was monitored at 430 nm immediately.

Kinetic Parameters and Stoichiometry of Inactivation of the Catalytic Subunit by Affinity Peptides **4** *and* **5**. A series of kinetic experiments was carried out with peptides **4** and **5** to determine whether their inactivation of the catalytic subunit obeys saturation kinetics. The experimental conditions were as described before (vide supra), except that concentrations of the inactivators were varied between 3 and 10 mM. Double-reciprocal plots of the observed first-order inactivation rate constants versus inactivator concentrations were linear within the concentration range employed. For the protection experiments, the enzyme was incubated with 10 mM $MgCl_2$ and 2 mM ATP or 10 mM $MgCl_2$, 2 mM β,γ-methylene-ATP, and 4 mM peptide **1** for 5–10 min at 35 °C prior to addition of the inactivator. The remaining enzymatic activity was assayed as described above.

To determine the stoichiometry of inactivation, a 5.0 mM solution of a given radioactive inactivator (specific activity ~2000 dpm/nmol) was allowed to react with 2 mg of catalytic subunit in 50 mM MOPS, 1 M KBr, and 0.2 mM DTT, pH 7.6 (600-μL total volume) at 35 °C. Within ~1.5–2.0 h of incubation less than 10% activity remained with concomitant precipitation of some inactivated enzyme. The inactivation mixture was filtered through a Centricon 30 (Amicon) microfiltration device by centrifugation (30 min, 4600*g*), and the retentate was washed with 500 μL of inactivation buffer followed by recentrifugation (6×). At the end of the washes, negligible amounts of radioactivity filtered through the Centricon device. The inactivated protein was taken up in 50 mM NH_4HCO_3 containing 1 mM $CaCl_2$ (pH 8.5, 1.2 mL). The radioactivity was measured in duplicate for 20-μL aliquots of the inactive enzyme solution.

Identification of the Sites of Modification in the Catalytic Subunit. The solution of 2 mg of inactivated catalytic subunit in 50 mM NH_4HCO_3 and 1 mM $CaCl_2$, pH 8.5 (vide supra), was incubated with 40 μg of TPCK-treated trypsin and 60 μg of *S. aureus* V8 protease at room temperature with gentle stirring for 4–5 h. The solution was subsequently passed through a Centricon 10 device (Amicon) by centrifugation (60 min, 4600*g*). Over 90% of the radioactivity was found in the filtrate. The filtrate was initially purified by HPLC (Vydac, ODS, 4.6 mm × 25 cm, 230 nm) using a gradient of 5–95% methanol in 150 mM sodium perchlorate and 50 mM sodium phosphate, pH 2.6. The radioactive fractions were concentrated in vacuo and were further purified isocratically on the same column using acetonitrile in 0.1% TFA.[1] Fractions

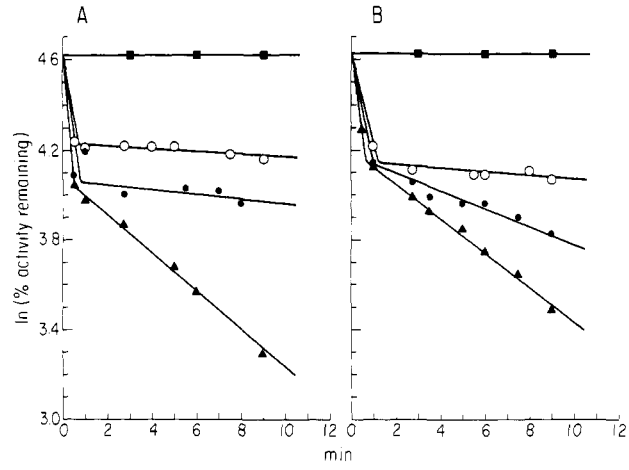


FIGURE 1: Kinetic experiments with peptide inactivators **4** (A) and **5** (B). Time-dependent inactivation of the catalytic subunit in the presence of 2.5 mM inactivator (▲); loss of activity in the presence of 2.5 mM inactivator, 10 mM $MgCl_2$, 2 mM β,γ-methylene-ATP and 4 mM peptide **1** (●); loss of activity in the presence of 2.5 mM inactivator, 10 mM $MgCl_2$ and 2 mM ATP (○); activity of the catalytic subunit incubated in 50 mM MOPS containing 1 M KBr and 0.2 mM DTT (pH 7.6) at 35 °C (■). Experimental conditions are given in the text.

corresponding to the purified peptide fragments were evaporated in vacuo to near dryness and then were individually taken up in a solution of 250 mM hydroxylamine in 500 mM MOPS buffer, pH 7.8 (1.0 mL). The solutions were stirred for 14–16 h at room temperature. Subsequently, the entire solution from each reaction was loaded on the same Vydac HPLC column, and the peptide fragments were eluted isocratically by using the acetonitrile–0.1% TFA medium described before.

The purified fragments were sequenced by Edman degradation according to the method of Hunkapillar et al. (1983) on an Applied Biosystems gas-phase sequenator. Typically, the quantities of peptide subjected to the sequencing cycles were within the 1.5–2.0 nmol range. The entire sequencing for the fragments obtained from the catalytic subunit inactivated by peptide **4** was done twice. The two experiments were carried out by using different preparations of both the inactivator and enzyme.

RESULTS

Inactivation of the Catalytic Subunit by Peptides **4** *and* **5**. Under conditions of inhibitor in excess, peptides **4** and **5** inactivated the catalytic subunit by a time-dependent process that followed apparent biphasic first-order kinetics. A rapid inactivation process, which resulted in ~40% loss of activity, was followed by a slower inactivation phase that accounted for the loss of the remaining enzyme activity (Figure 1). The inactivation process appeared to be irreversible, since the activity was not restored subsequent to removal of the inactivator from the enzyme solution.

Incubation of the catalytic subunit with $Mg^{II}ATP$ (2 mM)[2] or Mg^{II}-β,γ-methylene-ATP (2 mM) in the presence of peptide **1** (4 mM) afforded nearly complete protection from inactivation for the slow phase by 2.5 mM peptide **4** (Figure 1A). Protection was also observed for the slow phase of the inactivation by 2.5 mM peptide **5** (Figure 1B) under the same conditions. From these experiments at 2.5 mM inactivator concentration it was not clear whether any protection was observed for the fast phase. However, a set of similar experiments conducted at 0.4 mM inactivator concentration

[1] The appropriate concentration of acetonitrile was usually ~20% below the concentration of methanol at which the fragments eluted during the gradient elution.

[2] Peptides **4** and **5** were not phosphorylated by Mg[γ-^{32}P]ATP at a concentration of 2.0 mM as monitored according to the method of Kemp et al. (1977a).

indicated that no protection was afforded by $Mg^{II}ATP$ or a combination of Mg^{II}-β,γ-methylene-ATP and peptide **1** for the fast phase (data not shown). As shown in Figure 1, the catalytic subunit remained fully active in the absence of the inactivators for the duration of the kinetic experiments.

Equation 1 may be proposed as a minimal kinetic scheme for inactivation in the slow phase by our peptide affinity inactivators, giving the rate expression described by eq 2, under

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_2} E_{inact} \quad (1)$$

$$v = \frac{k_2[I]_0}{K_I + [I]_0}([E]_0 - [E_{inact}]) \quad (2)$$

conditions of inhibitor in excess. To demonstrate that enzyme inactivation obeys saturation kinetics for the slow phase, a series of kinetic experiments was carried out at different inactivator concentrations (3–10 mM) while the same enzyme concentration (10 μM) was maintained. Double-reciprocal plots of the inactivation rate constants as a function of the inactivator concentrations were linear for the slow phase of the inactivation process (data not shown). The inactivation rate constants (k_2) were determined to be 0.36 and 0.28 min^{-1}, while the dissociation constants (K_I) were 5.8 and 3.9 mM for peptides **4** and **5**, respectively.

Stoichiometry of Inactivation and Identification of the Modified Amino Acid Residues. Radioactive peptides were incubated with the catalytic subunit (2.0 mg) until more than 90% inactivation resulted. The excess inactivator was removed from the protein by use of a Centricon 30 ultrafiltration device. The stoichiometry of incorporation of the radioactive peptide during the enzyme inactivation process was determined by scintillation counting of an aliquot of the protein solution and normalizing the counts obtained to 100% inactivation. The results indicated an incorporation of 2.18 and 1.95 peptides per each molecule of the catalytic subunit for peptides **4** and **5**, respectively. An aliquot of the inactivated protein from experiments with each inactivator was treated overnight with 250 mM hydroxylamine in 500 mM MOPS buffer (pH 7.8) at room temperature. After filtration through Centricon 30, approximately 30% of the original radioactivity was found in the filtrate. This information indicated that both inactivators modified at least one acidic amino acid via an ester linkage, labile to hydroxylamine.

Proteins inactivated with radioactive peptides were proteolyzed by using *S. aureus* V8 protease and TPCK-treated trypsin for 4–5 h at room temperature. The radioactive peptide fragments were purified initially on an octadecylsilyl analytical column using a methanol/perchlorate–phosphate linear gradient. Two radioactive fragments were isolated in each case (Figure 2). The radioactive peptide fragments were desalted and purified further isocratically by using acetonitrile in 0.1% trifluoroacetic acid (TFA) as the elution medium. At this stage, the fragments were pure as judged by their HPLC traces. Since an ester bond would not survive the acidic conditions of the Edman degradation cycles during the peptide sequencing, the fragments were stirred with a solution of hydroxylamine to aminolyze the ester linkages to the more stable hydroxamic acid derivatives. After aminolysis, each fragment was purified by the same isocratic systems described above to afford pure fragments for peptide sequencing. The peptide fragments that eluted at 21.5 and 19.5 min during the gradient elution (parts A and B of Figure 2, respectively) were no longer radioactive after the hydroxylamine reaction. This observation is consistent with the hypothesis that the modified acidic residues were present in these fragments. On the other hand, the other two fragments retained their radioactivity

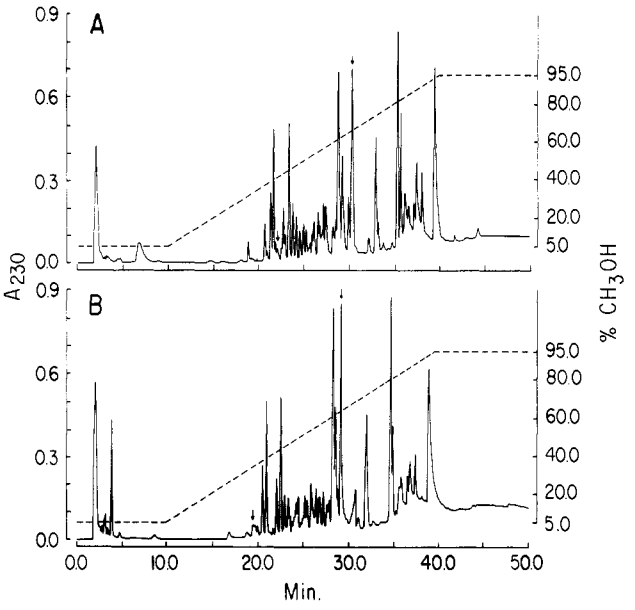


FIGURE 2: High-performance liquid chromatography of the proteolyzed catalytic subunit inactivated with radioactive peptides **4** (A) and **5** (B). Arrows indicate the radioactive peptide fragments. Experimental conditions are given in the text.

Table I: Proteolytic Peptide Fragments and the Sites of Alkylation[a]

inactivator	sequenced fragments[b,c]	sites of alkylation
4	TW*T*LCGTPE	T-197
	CGK*E*.....	E-346
5	TWTL*C*GTPE	C-199
	CGK*E*.....	E-346

[a] The one-letter notation for amino acids conforms with suggestions cited by the IUPAC–IUB Commission on Biochemical Nomenclature [(1968) *J. Biol. Chem. 243*, 3557]. [b] The cysteine residues were identified as their cysteic acid derivatives. [c] The residue alkylated is printed in italic type.

subsequent to the hydroxylamine reaction.

The peptides were sequenced by Edman degradation. The sequences of the fragments and their sites of modification are shown in Table I. Compound **4** modified Thr-197 and Glu-346, while compound **5** alkylated Cys-199 and Glu-346.

Discussion

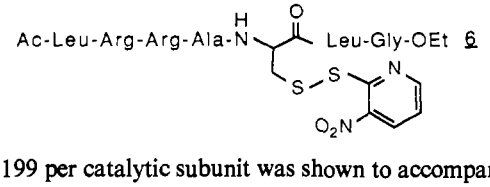
The catalytic subunit of the cAMP-dependent protein kinase has been the subject of many recent studies. Although the pathways by which the enzyme-catalyzed phosphorylation occur and the conformations of the peptide and metal ion–nucleotide complexes at the active site of the enzyme have been the subjects of extensive studies, relatively little is known about the amino acid residues involved in substrate recognition and in catalysis. Therefore, we have undertaken to identify active-site residues by using a series of peptide-based affinity and photoaffinity analogues. Initially, we prepared affinity peptides based on the sequence of the heptapeptide **1**, Kemptide, which is an excellent substrate for the catalytic subunit. The two arginine residues in **1**, which are important for substrate recognition by the enzyme, were substituted in turn with electrophilic N^{δ}-(bromoacetyl)ornithine residues. The resulting peptides (**4** and **5**) were effective inactivators for the catalytic subunit. Under conditions of inhibitor in excess, both peptides inactivated the catalytic subunit in a biphasic manner (Figure 1). Studies with radioactive preparations of **4** and **5** showed that fully inactivated enzyme was incorporated with two peptide moieties per each catalytic subunit. This implies that two protein modification events took place, one during the fast phase and the other during the slow phase of the inactivation

process. The first alkylation event resulted in ~40% loss of activity, whereas the second alkylation accounted for the loss of the remainder of the activity. In addition, protection experiments with $Mg^{II}ATP$, as well as Mg^{II}-β,γ-methylene-ATP and peptide **1**, indicated that the inactivation during the slow phase was active site directed, whereas the loss of activity during the fast phase was not. The peptide inactivators do not bind the enzyme well, as reflected by K_I values in the millimolar range (5.8 and 3.9 mM). This was expected, since substitution of the arginine residues with nonpositively charged amino acids produces poor substrates for the enzyme (Bramson et al., 1983). Nonetheless, the first-order inactivation constants (k_2) were quite respectable (0.36 and 0.28 min^{-1} for peptides **4** and **5**, respectively).

We observed that approximately 30% of the radioactivity was displaced when inactivated protein was allowed to react with hydroxylamine. This indicated the presence of at least one amino acid residue modified via an ester linkage. Because of the inherent lability of depsipeptides in aqueous media, it became imperative that subsequent manipulations of the modified protein be carried out in as short a time as possible. In addition, the esters had to be derivatized to give more stable analogues or be reduced to hydroxyl derivatives to survive the sequencing cycles. We decided on the former approach, since our attempt at ester reduction with borotritiide failed. Our decision to derivatize the ester was further supported by knowledge that reduction of esters in aqueous media typically requires specialized reagents that are not always commercially available in the radioactive form [e.g., see Bounds and Pollack (1987)]. After purification of the radioactive peptides obtained on proteolysis, the fragments were aminolyzed with hydroxylamine. Hydroxylaminolysis of esters is a facile reaction. In addition, only Asn–Gly bonds in peptides have been reported to be labile to this reaction under nonforcing conditions (Bornstein & Balian, 1977). The primary sequence of the catalytic subunit lacks any Asn–Gly sequence (Shoji et al., 1981); therefore, aminolysis with hydroxylamine seemed applicable to our needs. After hydroxylaminolysis, the fragments were purified and were sequenced by Edman degradation.

The sites of alkylation in the modified peptides may be deduced in two different ways. First, observation of an unknown peak in the HPLC trace for the phenylthiohydantoin of a given residue during the sequencing cycles may pinpoint the modified residue. Alternatively, a sharp drop in sequencing yield and/or a sequence termination, where a proteolytic cleavage would not be expected, may indicate the site of alkylation. The sites of alkylation in the larger fragments (i.e., Thr-197 and Cys-199) were established by the observation of unknown peaks during the sequencing of the nonapeptides and comparison of these sequences to those derived from proteolysis of the unmodified protein kinase. On the other hand, the modified residue in the smaller fragments (i.e., Glu-346) was identified by observation of an unknown peak in the HPLC trace accompanied by an abrupt yield drop and sequence termination at the site of alkylation.

Earlier work with affinity peptide **6**, which undergoes disulfide interchange with the sulfhydryl groups in the catalytic subunit, identified Cys-199 as the site of labeling (Bramson et al., 1982). Furthermore, attachment of one peptide residue



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to Cys-199 per catalytic subunit was shown to accompany *total* loss of activity. The labeling of cysteine was also shown to be inhibited in the presence of the substrate(s). These observations are consistent with the slower inactivation phase seen with peptides **4** and **5** as being due to alkylation of Thr-197 and Cys-199, respectively. This assertion is based not solely on the stated precedent for the active-site-directed modification of Cys-199 (Bramson et al., 1982) but also on the observation that modification during the fast phase does not inactivate the enzyme beyond ~40% loss of activity. Protection by the substrate(s) is observed for the slow phase, thereby placing Thr-197 and Cys-199 at or near the active site of the enzyme.

Table II: Sites of Autophosphorylation in Protein Kinases

protein kinases	sites of autophosphorylation
cAPK[a]	..TW*T*LC..[d]
cGPK[b]	..TW*T*FC..
pp60$^{v\text{-}src}$[c]	..NE*Y*TA..
p90$^{gag\text{-}yes}$	..NE*Y*TA..
P70$^{gag\text{-}fgr}$	..NE*Y*NP..
P120$^{gag\text{-}abl}$	..DT*Y*TA..
P140$^{gag\text{-}fps}$	..GV*Y*AS..
P85$^{gag\text{-}fes}$	..GV*Y*AA..
P68$^{gag\text{-}ros}$	..ND*Y*YR..
gp68/741$^{v\text{-}erb\text{-}B}$	..KE*Y*HA..

[a] The catalytic subunit of the cAMP-dependent protein kinase. [b] Cyclic GMP-dependent protein kinase. [c] The rest of the listed proteins are viral tyrosine protein kinases (Hunter & Cooper, 1985). [d] Site of autophosphorylation is printed in italic type.

The secondary structural predictions have subdivided the catalytic unit into three approximately equal-sized domains. The C-terminal domain is rich in acidic residues and has been postulated to be involved in substrate recognition (Shoji et al., 1981). This assertion was made primarily on the basis of the kinase requirement for a cluster of two positive charges on the N-terminal side of the sites of phosphorylation in both protein and peptide substrates. It seems reasonable to us that this acid-rich domain may be exposed near the protein surface to facilitate substrate recognition. Indeed, Glu-346—a residue near the C-terminal end of the acid-rich domain—may have been alkylated with peptides **4** and **5** because this residue is located on the path taken by the inactivators en route to the active site. Alkylation at this locus is clearly not protected in the presence of the substrate(s) and does not result in protein inactivation beyond approximately 40% loss of activity.

It has been suggested that some of the protein kinases, both serine/threonine and tyrosine kinases, might share an underlying structural basis for their analogies in function (Lincoln & Corbin, 1977; Hunter & Cooper, 1985). Stretches of approximately 260 amino acid residues show strong homologies in these proteins, despite their divergent strict specificities for their preferred sites of phosphorylation. It is noteworthy that Thr-197 and Cys-199 in the catalytic subunit of cAMP-dependent kinase are two residues that are conserved in the cGMP-dependent kinase (Thr-516 and Cys-518); both proteins are serine/threonine phosphotransferases (Table II). In addition, both Thr-197 and Thr-516 are sites of autophosphorylation in these two proteins. It is intriguing that the structural function of the Thr-197 and Thr-516 is "conserved" in a number of tyrosine kinases in the sense that a substitution of threonine for tyrosine is made in the homologous positions, allowing for these tyrosines to serve also as sites of autophosphorylation (Table II). While the implications for the existence of the autophosphorylation sites in protein kinases remain obscure at present, it has been speculated that autophosphorylation may play a role in modulation of substrate specificity or regulation of activity (Rangel-Aldao & Men-

delsohn-Rosen, 1976; Shoji et al., 1979) and for viral tyrosine kinases the ability to induce tumor formation (Snyder & Bishop, 1984) through conformational changes in protein. Indeed, it would be revealing to see whether the autophosphorylation sites in tyrosine kinases may also be alkylated by affinity inactivators designed specifically for these proteins.

In summary, we have developed two peptide-based affinity inactivators that inactivate the catalytic subunit of cAMP-dependent protein kinase in a biphasic manner. Sequencing of the alkylation sites, in conjunction with kinetic analyses, places residues Thr-197 and Cys-199 at or near the active site. We are currently studying the interaction of a number of other peptide-based affinity and photoaffinity analogues with the catalytic subunit. Photoaffinity peptides are being used with the expectation that the photogenerated radicals will alkylate nonnucleophilic residues in the active site (Miller & Kaiser, 1986; Miller and Kaiser, unpublished results). The collective results from our studies with all of these inactivators will help in elucidating a model for the residues comprising the active site and the recognition site of the cAMP-dependent protein kinase.

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Registry No. **2**, 113924-41-7; **3**, 113924-42-8; **4**, 113924-43-9; **5**, 113924-44-0; Thr, 72-19-5; Cys, 52-90-4; Boc-Gly, 4530-20-5; protein kinase, 9026-43-1.

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